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Matrix assembly of recombinant fibronectin polypeptide consisting of amino-terminal 70 kDa and carboxyl-terminal 37 kDa regions

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Three different forms of recombinant human fibronectin polypeptides consisting of the amino-terminal 70 kDa region, the carboxyl-terminal 37 kDa region, or both, were expressed in mouse L cells. Although either the amino-terminal or the carboxyl-terminal region alone was only poorly incorporated into the extracellular matrix, the fused form of the polypeptide was highly capable of assembling into the matrix. These results indicate that matrix assembly of fibronectin requires both regions and can proceed in the absence of most of the type III repeats including the one containing the cell adhesive Arg-Gly-Asp sequence.

Fibronectin; Extracellular matrix; Fibrillogenesis

1. INTRODUCTION

Fibronectin (FN) is a glycoprotein present in the extracellular matrix and various body fluids [1,2]. FN synthesized and secreted by cells is incorporated into the extracellular matrix through a complex cell-mediated process [3]. Although the molecular mechanism of FN matrix assembly is as yet only poorly understood, the amino-terminal 70 kDa region and the central cell-binding domain have been inplicated in the assembly process [4,5].

Recently, we have shown that the carboxyl-terminal 37 kDa fibrin-binding domain of human FN retained the ability to dimerize when expressed in mouse L cells, but it was barely incorporated into the extracellular matrix of the cells [6]. Given the suggested role of the amino-terminal 70 kDa region in the FN matrix assembly, we expressed in the present study the carboxyl-terminal 37 kDa domain in a fused form with the amino-terminal 70 kDa region (hereafter referred to as 'r70F2'; see Fig. 1) and compared the ability of the recombinant protein to assemble into the extracellular matrix with that of other recombinant proteins consisting of either the amino-terminal 70 kDa ('r70') or the carboxyl-terminal 37 kDa ('rF2') region alone.

2. MATERIALS AND METHODS

2.1. *DNA construction*

A cDNA clone pCF26 encoding the signal sequence and the amino-terminal 70 kDa region of human FN was prepared by oligonucleotide-primed cDNA synthesis [7] using the poly(A)$^+$ RNA isolated from SV40-transformed human fibroblast WI-38VA13 as a template. A 1,582 bp *Sma*I–*Sal*I fragment encoding the amino-terminal 70 kDa region was amplified from pCF26 using the polymerase chain reaction and inserted to the *Sma*I/*Nsp*V-cleaved rF2 expression vector pAISF21 [6], together with either the 1,510 bp *Xho*I–*Nsp*V fragment of pHCF2D [6] encoding the carboxyl-terminal 37 kDa region of human FN or the 274 bp *Sal*I–*Nsp*V fragment of pHCF2D encoding only the carboxyl-terminal 14 amino acid residues.

2.2. *DNA transfection and selection of stable transformants*

Each expression vector described above was co-transfected to mouse L cells along with a selection marker pKO_{neo} by the calcium-phosphate precipitation method [8]. G418-resistant colonies were screened for the expression of recombinant FN mRNAs by Northern blot analysis. Clones expressing the highest levels of the recombinant FN transcripts were used in the subsequent immunoprecipitation and immunofluorescence experiments.

2.3. *Immunoprecipitation*

Mouse L cells were pulse-labeled with [^{35}S]methionine (American Radiolabeled Chemicals, MO) as described in [6]. Radiolabeled cells were solubilized with 20 mM Tris-HCl, pH 8.0, containing 1.5% sodium deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2 mM *N*-ethylmaleimide with or without prior treatment with 2.5% TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin at 37°C for 5 min, followed by the addition of a large excess of soybean trypsin inhibitor. The cell lysates were immunoprecipitated with monoclonal anti-human FN antibody FN9-1 or FN8-12 immobilized on Sepharose CL4B (generous gifts from Mr. Masahiko Katayama, Takara Shuzo, Japan) and the precipitates were analyzed by SDS-PAGE and subsequent fluorography.

2.4. *Indirect immunofluorescence staining*

The recombinant FN polypeptides incorporated into the extracellular matrix were visualized by indirect immunofluorescence using monoclonal anti-human FN antibodies FN9-1 and FN8-12 (Takara Shuzo, Japan) or polyclonal anti-human FN antibody as described in [6].

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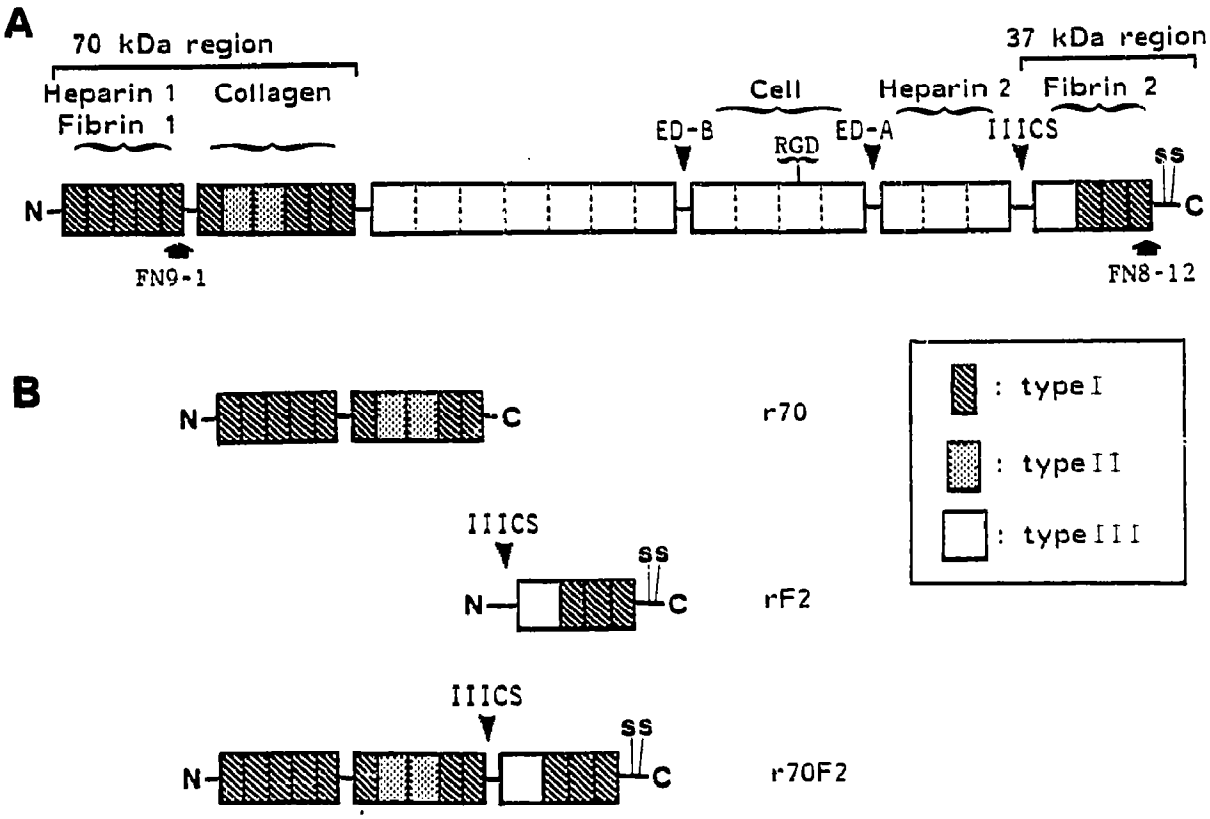

Fig. 1. Schematic diagram of FN (A) and FN-derived recombinant polypeptides (B). FN consists of six functional domains with binding specificities toward heparin, fibrin, collagen, and cell surface integrins. These domains are made up with three types of internal homology repeats named types I, II, and III. It should be noted that the recombinant polypeptides lack most of the central type III repeats.

3. RESULTS AND DISCUSSION

The L cell transformants, L/70, L/201, and L/70F2, stably expressing r70, rF2, and r70F2, respectively, were pulse-labeled with [^{35}S]methionine and the recombinant polypeptides were immunoprecipitated from both cell lysates and the culture supernatants after a 6 h chase. Almost all of the radiolabeled r70 was secreted to the medium and barely retained on the cell surface or within cytoplasm (Fig. 2, lanes 1–3). Similary, rF2 was predominantly secreted into the medium as differentially glycosylated heterogeneous polypeptides of 60–90 kDa, leaving a small fraction associated with cells as 66 kDa, 54 kDa, and 51 kDa polypeptides (Fig. 2, lanes 4–6; see also [6]). Most of the cell-associated rF2 polypeptides were degrated when the cells were treated with trypsin, suggesting that they were exposed at the cell surface.

In contrast, a significant amount of r70F2 was immunopresopitated from the cell lysate (Fig. 2, lines 7–9). Trypsin treatment of L/70F2 cells markedly reduced the amount of the cell-associated 70F2, indicating that most of the 70F2 recovered in the cell lysate was present on the cell surface. SDS-PAGE of the unreduced r70F2 showed that r70F2 was secreted into the medium as two major forms with molecular masses of 350 kDa and 200 kDa (Fig. 2, lane 10). Since a significant amount of mouse FN was co-precipitated with r70F2, this was considered to be the heterodimer of r70F2 linked to mouse FN and the homodimer of r70F2. These results indicated that r70F2 expressed in mouse L cells retained the ability to demerize and was capable of assembling into the extracellular matrix.

Assembly of these recombinant polypeptides into the extracellular matrix was further examined by immunofluorescence staining of the stably transformed L cells with monoclonal antibodies, FN9-1 and FN8-12, which specifically recognize the amino-terminal and the carboxyl-terminal regions of human FN, respectively (Fig. 3). Polyclonal anti-FN antibody was also used to visualize the whole FN matrix consisting of both endogenous mouse FN and recombinant human FN polypeptides. In spite of the presence of well-developed mouse FN matrix, r70 was not detectable on the surface of L/70 cells (Fig. 3, A–C). Similarly, the matrix of L/F2 cells expressing the carboxyl-terminal 37 kDa region was only weakly stained with FN8-12 but not with FN9-1 (Fig. 3, D–F). The matrix of L cells expressing r70F2

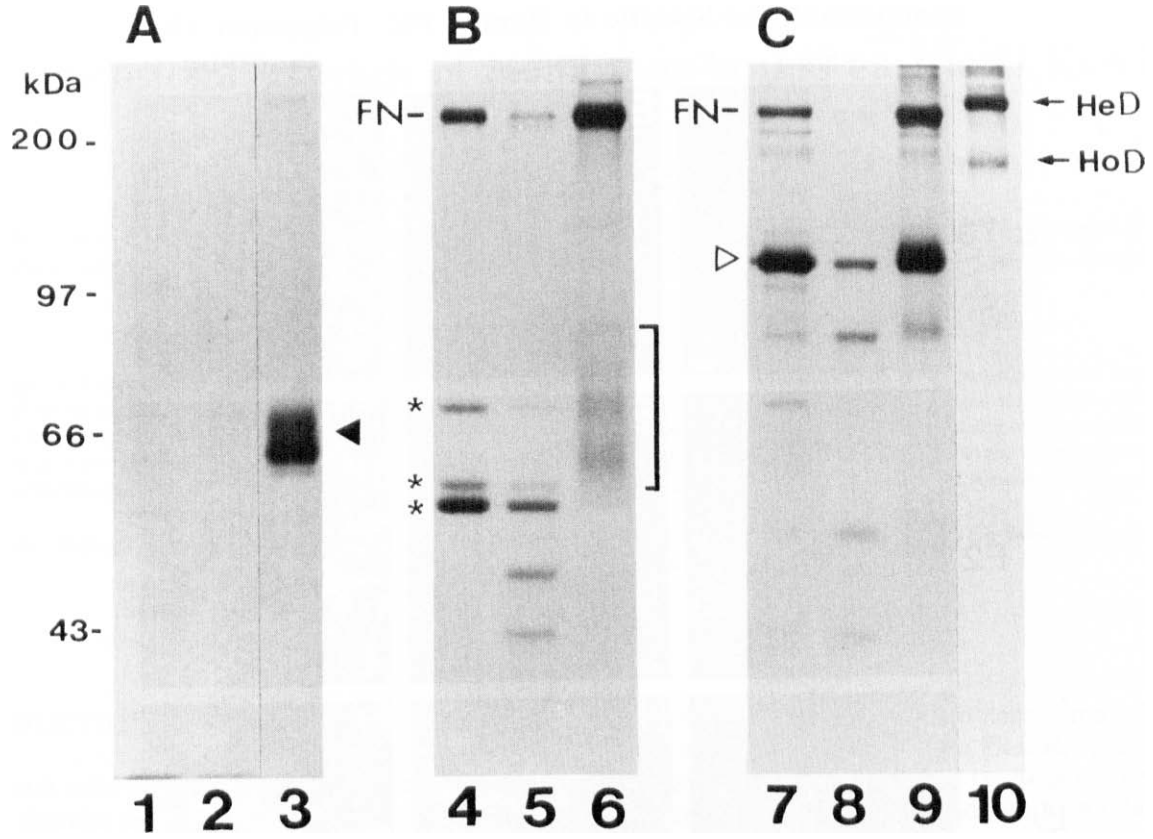

Fig. 2. Immunoprecipitation of recombinant polypeptides expressed in mouse L cells. The cells expressing r70 (A), rF2 (B), or r70F2 (C) were metabolically labeled with [^{35}S]methionine and chased with a large excess of cold methionine for 6 h. The labeled recombinant polypeptides associated with cells before (lanes 1, 4, 7) or after trypsin treatment (lanes 2, 5, 8) as well as those secreted into the medium (lanes 3, 6, 9, 10) were immunoprecipitated with monoclonal antibody specific to human FN. Immunoprecipitated polypeptides were analyzed by SDS-PAGE under reduced (lanes 1–9) or unreduced (lane 10) conditions. r70F2 exists as a homodimer (HoD) as well as a heterodimer linked to mouse FN (HeD). Open and closed arrowheads and bracket indicate the position of the secreted recombinant polypeptides. Asterisks indicate the putative precursor forms of rF2.

was, however, strongly positive for immunostaining with both FN9-1 and FN8-12 (Fig. 3, G–I), confirming that r70F2 was capable of assembling into the extracellular matrix. These results, taken together with the observations with immunoprecipitation of pulse-labeled recombinant polypeptides, indicated that both the amino-terminal 70 kDa and the carboxyl-terminal 37 kDa regions are necessary for incorporation of FN into the extracellular matrix.

Monoclonal antibodies directed to the cell-binding domain have been shown to inhibit incorporation of FN into matrix [5,9], suggesting that surface fibronectin receptor, i.e $\alpha 5\beta 1$ integrin, is involved in FN matrix assembly. Our observation that r70F2 was capable of assembling into the matrix, however, indicates that interaction of FN with $\alpha 5\beta 1$ integrin is not prerequisite for FN matrix assembly, since r70F2 is devoid of almost all of the FN type III repeats including the one containing the cell adhesive RGD sequence.

FN matrix assembly is considered to be a process consisting of two consecutive events, i.e. nucleation and elongation of FN fibrils. Since mouse L cells produce an elaborated FN matrix, the incorporation of r70F2 into the extracellular matrix may not require nucleation of the 70F2 fibrils and proceed only through the elongation event, i.e. addition of r70F2 to the pre-existing mouse FN fibrils. The interaction of FN with $\alpha 5\beta 1$ integrin may well be important in the nuceation of FN fibrils.

After the completion of this work, Schwarzbauer [10] reported that the carboxyl-terminal half of rat FN became capable of assembling into the extracellular matrix when expressed in mouse SV12 cells as a fusion polypeptide with the amino-terminal 70 kDa region. Our results are in good agreement with this observation and further narrow-down the minimal carboxyl-terminal region required for FN matrix assembly to the fibrin-binding 37 kDa region.

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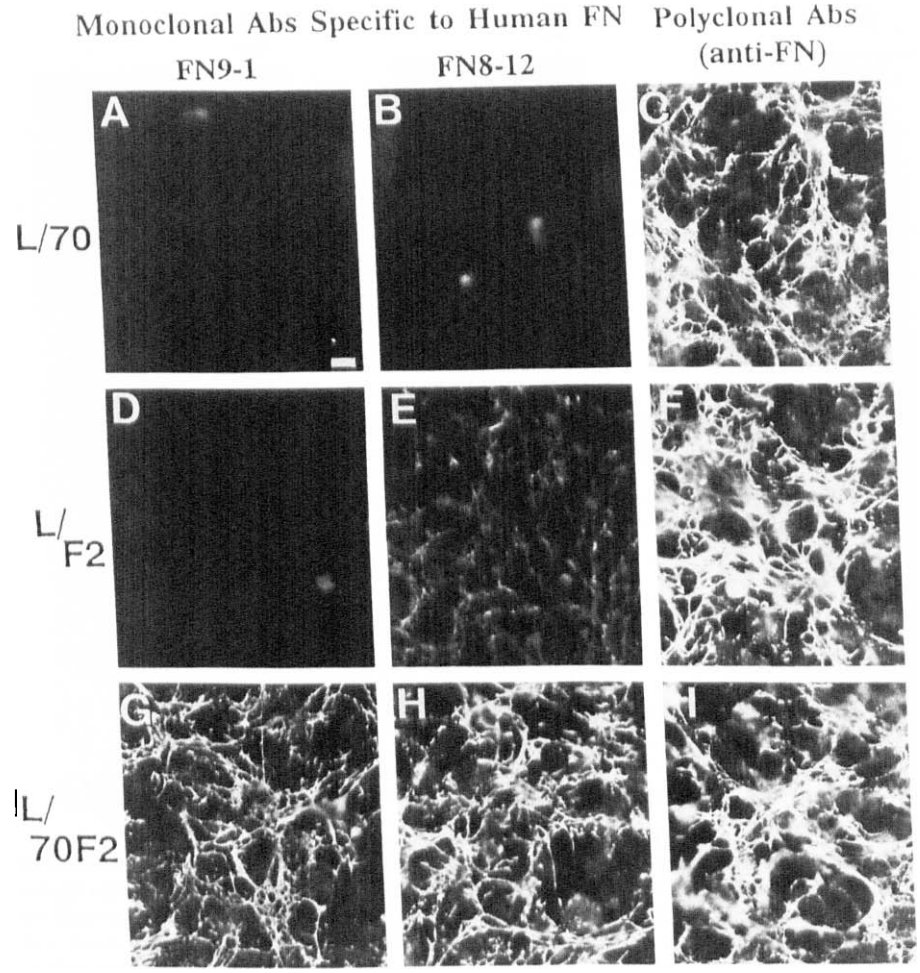

Fig. 3. Immunofluorescence staining of the FN matrix of stable transformants. Mouse L cells expressing r70 (A–C), rF2 (D–F), and r70F2 (G–I) were stained with monoclonal anti-human FN antibodies FN9-1 (A, D, G) and FN8-12 (B, E, H) as well as with polyclonal anti-FN antibody (C, F, I). Scale bar = 10 μm.

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